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(54) Title: ISOLATING NUCLEIC ACID

(57) Abstract: A process. for isolating nucleic acid from a nucleic acid containing sample, which comprises: (a) providing a chaotrope; (b) providing a nucleic acid binding solid phase capable of binding nucleic acid in the presence of the chaotrope; (c) contacting the sample with the nucleic acid binding solid phase in the presence of a liquid phase comprising the chaotrope; and (e) optionally separating the solid phase with the nucleic acid bound thereto from the liquid phase, wherein the solid phase bears acid groups on its surface.

ISOLATING NUCLEIC ACID

Field of the Invention

The present invention relates to a process for isolating nucleic acid from a nucleic acid-containing sample, and to a kit therefor.

Background to the Invention

Procedures involving nucleic acids such as DNA and RNA continue to play a crucial role in biotechnology. Nucleic acid detection and manipulation including hybridisation, amplification, sequencing and other processes generally require nucleic acid to have been isolated from contaminating material. Where a nucleic acid-containing sample is a biological sample, contaminating material may include proteins, carbohydrates, lipids and polyphenols. Accordingly, a variety of approaches have hitherto been used in the isolation of DNA or RNA.

Early methods of isolating nucleic acid involved a series of extractions with organic solvents, involving ethanol precipitation and dialysis of the nucleic acids. These early methods are relatively laborious and time-consuming and may result in low yield. Isopropanol may also be used in the precipitation of the nucleic acid.

An alcohol precipitation method is described in US5523231. Nucleic acid is precipitated by highly concentrated alcohol in the presence of magnetic beads. According to this disclosure, use of an alcohol such as ethanol or isopropanol at around 70% (v/v) causes nucleic acid to precipitate around the magnetic beads but not to bind to

the bead. The precipitate can be separated from supernatant by the application of a magnetic field. The nature of the magnetic beads is not critical, with cellulose/ferric oxide beads being used experimentally.

US5234809 describes a procedure to isolate DNA from biological samples which uses a chaotropic agent together with a silica based nucleic acid binding solid phase. Guanidine hydrochloride at pH 3 to 5 or guanidine thiocyanate at higher pH, combined with other salts, is used as the chaotropic agent. After binding of the DNA to the solid surface, the solid phase may be washed with the chaotropic agent to remove any biological contamination followed by treatment with 70% ethanol to remove the chaotrope. The DNA is eluted using water.

A variant on this methodology is described in US6027945. Here, a method is described which also uses a silica-based nucleic acid binding solid phase in the presence of a chaotrope to isolate nucleic acid. According to this method, the silica-based solid phase is magnetic, thereby facilitating separation of the solid phase containing the target nucleic acid from the liquid phase containing contaminants upon application of a magnetic field.

US 5990302 describes a method for isolating RNA which is also performed in the presence of a chaotrope. A sample is mixed with an acidic solution containing a lithium salt, a chaotropic agent and a nucleic acid-binding carrier to absorb the RNA onto the carrier. The RNA-bound carrier is isolated from the liquid phase and eluted. Magnetic silica particles are used as the nucleic acid-binding carrier,

although silica, cellulose, nitrocellulose, latex and hydroxyapatite are all mentioned as possible carriers.

WO96/18731 also uses magnetic particles to bind nucleic acid. In this disclosure the magnetic particles are polystyrene-based and polyurethane-coated and a detergent is used instead of a chaotrope.

US 5705628 discloses a method of separating polynucleotides, especially DNA, by binding the polynucleotides to a magnetic micro particle having a functional group-coated surface. Carboxyl groups are mentioned as the functional group and are coated on the surface of a silica based micro particle. This method requires the presence of salt and a polyalkylene glycol such as polyethylene glycol at a concentration in the range 7 to 13%.

Other types of magnetic particles are available from the agent suppliers for use not as a binding phase for isolating nucleic acid, but as a starting material for the production of affinity materials. For example, magnetic beads bearing on their surface carboxylic acid moieties and composed of a highly cross-linked polystyrene are known for use in coupling reactions with proteins, peptides, oligonucleotides or other target specific molecules. A bi-functional cross-linking reagent such as carbodiimide is first coupled to the carboxylic acid moieties and then used as a reactive group to couple the target specific molecules via primary amino groups.

In spite of the widespread use of silica-based nucleic acid binding solid phases, the use of a silica surface has its drawbacks in that it is difficult to control the synthesis and preparation of silica-based solid phases.

Summary of the Invention

In a first aspect, the present invention provides a process for isolating nucleic acid from a nucleic acid-containing sample, which comprises:

(a) providing a chaotrope;

(b) providing a nucleic acid binding solid phase capable of binding nucleic acid in the presence of the chaotrope;

(c) contacting the sample with the nucleic acid binding solid phase in the presence of a liquid phase comprising the chaotrope; and (d) optionally separating the solid phase with the nucleic acid bound thereto from the liquid phase, wherein the solid phase bears acid groups on its surface.

In a second aspect, the present invention provides a kit for isolating nucleic acid from a nucleic acid-containing sample, which kit comprises: (a) a chaotrope; and (b) a nucleic acid binding solid phase capable of binding nucleic acid in the presence of the chaotrope, wherein the solid phase bears acid groups on its surface.

It has surprisingly been found that a solid phase which bears acid groups on its surface is capable of non-specific or non-covalent direct binding of nucleic acids. Such solid phases are found to be as efficient as silica surfaces for the isolation of nucleic acids.

The present invention provides an advantage in that acid groups on the surface of the solid phase represent a more controlled chemistry for the synthesis and preparation of the solid phase. An acid surface, for example an organic acid surface such as a carboxylic acid surface is also more useful than the corresponding silica surface. Following non-specific nucleic acid isolation, covalent coupling of the nucleic acid to the acid may be effected *in situ*.

Typically, the concentration of acid groups on the surface should be above 1μmol/g solid phase, preferably above 10μmol/g and more preferably above 100μmol/g solid phase. The higher the density of the acid groups, the greater the surface charge on the solid phase.

It is preferred that the isoelectric point of the solid phase surace is below pH 8, preferably below pH 6 and more preferably below pH 4.

Among those acid groups useable according to the present invention may be mentioned carboxy, sulpho and aryloxy groups. For example, the carboxy or sulpho groups may be linked to the solid phase by alkylene or arylene groups so as to form carboxylic or sulphonic acids. Aryloxy groups such as phenoxy groups may also be so linked and may incorporate further aromatic or aliphatic moieties. Carbon atoms in each type of organic acid may be substituted with heteroatoms. The presence of such heteroatoms and the optional presence of further functional groups on the surface, including esters, amines, alcohols, carboxylic acids, amides, halides, aldehydes, ketones, imines, nitro compounds, thiols, thioesters, nitriles, acid anhydrides

and sulphonic compounds may each contribute to the properties of the solid phase, especially to the hydrophilicity of the solid phase. The preferred solid phase is hydrophilic because too hydrophobic a solid phase (for instance where there is too a high a concentration of polystyrene) will tend to give problems with nucleic acid binding.

In accordance with the present invention, the solid phase bearing acid groups on its surface may be prepared by various different methods. For example, a solid phase comprising a silica or non-silica surface may be modified by a reactant to introduce the acid groups. Such surfaces may be modified by carboxylic acids, for example by using an epoxy coating of silica followed by carboxylic introduction. In the absence of silica, for instance using hydroxyl coated surfaces, these may be transformed into a carboxylic acid coated surface by a variety of different means. In one example, acid anhydrides are introduced onto the hydroxyl-based surface. In another example, an epoxy coating of the hydroxyl moieties is added to the solid surface, followed by carboxylic acid introduction by various means.

It is preferred that the solid phase is an organic solid phase rather than one based on silica. Silicia-based solid phases tend to give poorer yields and are more difficult to synthesize. It is preferred, if starting from a silica-based surface, to apply an organic coating which includes acid groups or on which is subsequently introduced acid groups. It is therefore preferred that the solid phase is

substantially free of silica or at least free of surface-exposed silica.

The nucleic acid-containing sample typically comprises a biological sample such as a cellular sample. The biological sample may or may not need to be pretreated, depending on its structure. For example, in the case of plant or fungal cells or solid animal tissue, pretreatment would be required as is known in the art. Samples stored in the form of a solid phase such as a paraffin section may also need pretreatment. Samples may be from foodstuffs, environmental samples or clinical samples and may contain prokaryotic or eukaryotic cells or other moieties such as mycoplasmas, protoplasts or viruses. Blood products are an important area for nucleic acid isolation and the present invention is particularly applicable to whole blood and other blood products such as plasma, serum and buffycoat.

The nucleic acid to be isolated may be DNA, RNA or a modified form thereof. Where the nucleic acid is DNA, this may be ds or ss DNA. Where the nucleic acid is RNA, this may be rRNA, mRNA or total RNA.

The chaotrope generally comprises a chaotropic ion provided at a concentration sufficiently high to cause the nucleic acid to lose its secondary structure and, in the case of double-stranded nucleic acids, to melt. Chaotropes are thought to disrupt hydrogen-bonding in water so as to make denatured nucleic acid more stable than its undenatured counterpart. The chaotrope typically comprises a guanidinium salt, urea, or an iodide, chlorate, perchlorate

or (iso)thiocyanate. Preferred chaotropes include guanidinium thiocyanate, and guanidinium hydrochloride.

The concentration of chaotrope typically present when contacted with the sample is in the range 2M to 8M.

The form of the solid phase includes sheets, sieves, sinters, webs and fibres. Particles are particularly useful as these may be packed in a column or used in suspension and have high binding capacity. Magnetic particles are particularly preferred because of the ease with which they merely separated from an associated liquid phase in a magnetic field. Typical materials for use in magnetic particles include magnetic metal oxides especially the iron oxides. Useful magnetic oxides include iron oxides in which, optionally all or a part of the ferrous iron thereof is substituted with a divalent transition metal such as cadmium, chromium, cobalt, copper, magnesium, manganese, nickel, vanadium and/or zinc.

The step of separating the solid phase with the nucleic acid bound thereto from the liquid phase is generally required in order to remove contaminants in the liquid phase. Further washing steps may be applied to the solid phase at this point. Any conventional separation step for separating solid phase from liquid phase is applicable, including centrifugation and decanting of the liquid phase from the pelleted solid phase or using a column in which the solid phase is packed and the liquid phase passed through. Where the magnetic solid phase is used, this facilitates separation, which can be carried out in the presence of a magnetic field.

Depending on the form in which the isolated nucleic acid is required, a further elution step can be provided. In some cases it may be satisfactory for the nucleic acid to remain bound to the solid phase. This may be the case if further manipulations of the nucleic acid on a solid phase are required, such as an amplification step. Equally, the nucleic acid may be eluted from the solid phase by applying an elution solution, which may simply be water or a buffer.

Where the nucleic acid is required to remain associated with the solid phase, it is possible to couple the nucleic acid to the solid phase using a coupling reagent. Typical coupling reagents include bifunctional cross-linking agents such as carbodiimides e.g.(1-ethyl-3-(3 dimethylamino-propyl) or di(cyclohexyl) carbodiimide.

In a typical procedure, whilst the nucleic acid is still non-specifically attached to the solid phase, this may be washed with 70% ethanol and an alcohol soluble coupling reagent, such as one of those described above, may be added. Following incubation, typically at room temperature and a washing step, the nucleic acids may be coupled covalently to the solid phase.

Detailed Description of the Invention

The present invention is now described in more detail, by way of example only, with reference to the following Example.

Example

The magnetic particles. Hydrophilic magnetic particles are available from numerous sources (Dynal, Chemagen, MicroMod, ChemiCell etc). Any hydrophilic magnetic particle with, for instance, OH moieties can be transformed into magnetic particles with a surface carboxylic acid (COOH) functionality. In these experiments ca 15 mg of MPVA C22 Carboxylic Acid (Chemagen, Germany) with a COOH content of ca 900 umol/g) and ca 15 mg of Dynabeads M270 Carboxylic Acid (Dynal, Norway), with a COOH content of at least 150 umol/g were utilized.

The chaotropic lysis and binding solution 1. A 7.5M Guanidine hydrochloride (Sigma)/in 1M LiCl (Fluka) and 0.1 M potassium phthalate was adjusted to pH 2.5 with the aid of Hydrochloric acid (Merck).

Binding Solution 2. 8M LiCl (Sigma)

The wash I solution. To 7.5M guanidine hydrochloride (Sigma) in 0.8M LiCl was added 96% EtOH (3 volumes chaotrope: 1 volume EtOH).

The wash II solution. To 150 µl, 8M LiCl (Sigma) was added 750 ul 96% EtOH. To 600 ul of this solution was added 100 ul water.

The wash III solution. 10 mM NaCl.

The binding conditions. To cultured cells ($4x10^6$ HL_{60}) were added 700 ul of the chaotropic lysis and binding solution and 100 ul binding solution 2. The suspension was

"sheared" with a syringe. The solution was allowed to lyse for 10 min. The magnetic beads, as a water suspension, were added and allowed to incubate for 1 min. The beads were resuspended in 900 ul washing solution I and again collected on a magnet. The beads were resuspended and washed in 900 ul washing solution II and collected on a magnet. This was repeated with 900ul washing solution III. Finally, 100 ul water was added to the beads and they were resuspended at 69°C for 3 min. The beads were collected on a magnet and the supernatant was transferred to a new tube The yield of isolated RNA was measured on a Spectrophotometer (Perkin Elmer, Lambda EZ 201).

Solid Surface Chemistry	Ratio 260/280	Yield total RNA ($4x10^6$ HL60)
Silica	2.01	40ug
M270 CA	1.86	46ug
MPVA C22	1.86	40ug

CLAIMS:

1. A process for isolating nucleic acid from a nucleic acid-containing sample, which comprises:
(a) providing a chaotrope;
(b) providing a nucleic acid binding solid phase capable of binding nucleic acid in the presence of the chaotrope;
(c) contacting the sample with the nucleic acid binding solid phase in the presence of a liquid phase comprising the chaotrope; and
(e) optionally separating the solid phase with the nucleic acid bound thereto from the liquid phase, wherein the solid phase bears acid groups on its surface.

2. A process according to claim 1, wherein the concentration of acid groups is above 10 μmol/g solid phase.

3. A process according to claim 1 or claim 2, wherein the isoelectric point of the solid phase is below pH 6.

4. A process according to any of claims 1 to 3, wherein the acid groups comprise carboxy, sulpho or aryloxy groups.

5. A process according to any preceding claim, wherein the solid phase comprises an organic solid phase.

6. A process according to any preceding claim, wherein the solid phase is hydrophilic.

7. A process according to any preceding claim, wherein the solid phase is magnetic.

8. A process according to any preceding claim, wherein the chaotrope comprises a guandinium salt, urea, or an iodide, chlorate, perchlorate or (iso)thiocyanate.

9. A process according to any preceding claim, wherein the nucleic acid comprises DNA.

10. A process according to claim 9, wherein the DNA comprises ds or ss DNA.

11. A process according to any of claims 1 to 8, wherein the nucleic acid comprises RNA.

12. A process according to claim 11, wherein the RNA comprises rRNA, mRNA or total RNA.

13. A process according to any preceding claim, which further comprises a step of eluting the nucleic acid from the solid phase.

14. A process according to any preceding claim, wherein the sample comprises a biological sample.

15. A process according to claim 14, wherein the biological sample comprises a cellular sample.

16. A process according to claim 14 or claim 15, which further comprises a lysis step comprising subjecting the biological sample to conditions to lyse the sample.

17. A kit for isolating nucleic acid from a nucleic acid-containing sample, which kit comprises:

(a) a chaotrope; and

(b) a nucleic acid binding solid phase capable of binding nucleic acid in the presence of the chaotrope, wherein the solid phase bears acid groups on its surface.

18. A kit according to claim 17, wherein the concentration of acid groups is above 10 µmol/g solid phase.

19. A kit according to claim 17 or claim 18, wherein the isoelectric point of the solid phase is below pH 6.

20. A kit according to any of claims 17 to 19, wherein the acid groups comprise a carboxy, sulpho or aryloxy groups.

21. A kit according to any of claims 17 to 20, wherein the solid phase comprises an organic solid phase.

22. A kit according to any of claims 17 to 21, wherein the solid phase is hydrophilic.

23. A kit according to any of claims 17 to 22, wherein the solid phase is magnetic.

24. A kit according to any of claims 17 to 23, which further comprises a solution for eluting the nucleic acid from the solid phase.

25. A kit according to any of claims 17 to 24, which further comprises a lysis solution for lysing biological samples.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 03/01822

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 199 37 607 A (BILATEC GES ZUR ENTWICKLUNG BI) 15 February 2001 (2001-02-15) abstract page 2, line 53 page 4, line 32; claims ---	1-25
Y	US 5 705 628 A (HAWKINS TREVOR) 6 January 1998 (1998-01-06) abstract column 3, line 28 - line 55 ---	1-25
Y	US 6 027 945 A (SMITH CRAIG E ET AL) 22 February 2000 (2000-02-22) abstract; claims 1,12,13 --- -/--	1-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 03/01822

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00 69872 A (PROMEGA CORP) 23 November 2000 (2000-11-23) the whole document -----	1-25

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